



A thaumatin-like gene in nonclimacteric pepper fruits used as molecular marker in probing disease resistance, ripening, and sugar accumulation

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Abstract

During pepper (*Capsicum annuum*) fruit ripening, the ripe fruit interaction with the anthracnose fungus, *Colletotrichum gloeosporioides*, is generally incompatible. However, the unripe fruit can interact compatibly with the fungus. A gene, designated *PepTLP* (for pepper thaumatin-like protein), was isolated and characterized by using mRNA differential display. The *PepTLP* gene encodes a protein homologous to other thaumatin-like proteins and contains 16 conserved cysteine residues and the consensus pattern of thaumatin. *PepTLP* gene expression is developmentally regulated during ripening. The accumulation of *PepTLP* mRNA and *PepTLP* protein in the incompatible interaction was higher than that in the compatible one. Furthermore, *PepTLP* gene expression was stimulated by both jasmonic acid treatment and wounding during ripening, but by wounding only in the unripe fruit. Immunolocalization studies showed that it is localized to the intercellular spaces among cortical cells. The expression of the *PepTLP* gene upon fungal infection was a rise from the early-breaker fruit. The development of anthracnose became significantly prevented with beginning of fruit ripening, and the sum total of sugar accumulation increased. The results suggest that the *PepTLP* gene can be used as a molecular marker in probing for disease resistance, ripening, and sugar accumulation in nonclimacteric pepper fruits.

Introduction

Fruit ripening is a unique developmental process accompanied by considerable physical and biochemical changes in various plant species. These changes involve pigmentation, softness, and the accumulation of sugar and aroma (Giovannoni, 1993). Peppers are nonclimacteric fruits that do not have an ethylene burst during ripening. The ethylene burst is a characteristic of climacteric fruits, such as apple, peach, and tomato. Generally, ripe fruits that are rich in macromolecules are especially susceptible to pathogen infection. To protect the reproductive organ, pathogenesis-related (PR) and antifungal proteins are being highly accumu-

lated during fruit ripening in banana (Clendennen and May, 1997), cherry (Fils-Lycaon *et al.*, 1996), grape berries (Robinson *et al.*, 1997; Tattersall *et al.*, 1997; Salzman *et al.*, 1998), pepper (Meyer *et al.*, 1996; Oh *et al.*, 1999c), and tomato (Pressey, 1997).

To inhibit pathogen invasion, plants deploy hypersensitive reactions (Goodman and Novacky, 1994), deposition of lignin (Dean and Kúc, 1988), and oxidative cross-linking (Brisson *et al.*, 1994) as their first line of defense. After that, PR and antifungal proteins, such as chitinase (PR-3 family), thaumatin-like proteins (TLP) (PR-5 family), and cysteine-rich proteins become involved in the defense response. Especially, TLPs were seen to accumulate during the fruit ripening of bananas (Clendennen and May, 1997), cherries (Fils-Lycaon *et al.*, 1996), grapes (Tattersall *et al.*, 1997; Salzman *et al.*, 1998), and tomato (Pressey, 1997). Also, the

The nucleotide sequence data reported will appear in the GenBank Nucleotide Sequence Database under the accession number AF297646.

TLPs and TLP genes are being induced in various organs of plants upon pathogen infection (Ruiz-Medrano *et al.*, 1992; Rodrigo *et al.*, 1993), abiotic stress (Zhu *et al.*, 1993), and chemical stimuli (King *et al.*, 1988). Recently, a TLP that functions as antifreeze protein has been isolated from winter rye during cold acclimation (Hiilovaara-Teijo *et al.*, 1999; Yu and Griffith, 1999). Also, several TLPs are known to exhibit antifungal activity *in vitro* and some have recently been shown to have β -glucanase activity (Cheong *et al.*, 1997). Although the precise function of TLP is not yet understood, the antifungal activity of TLP appears to act by permeabilizing fungal membranes (Robert and Selitrennikoff, 1990).

Colletotrichum gloeosporioides causes severe anthracnose diseases especially in the unripe-green pepper fruit, but not in ripe-red fruit (Oh *et al.*, 1998). In the incompatible ripe fruit, the incidence of appressorium and infection hypha formation by the fungus was lower than in the compatible unripe fruit (Kim *et al.*, 1999; Oh *et al.*, 1999a). We, therefore, decided to look for a molecular mechanism that would explain the incompatibility of interactions during pepper fruit ripening. Several genes that may have an important role in the defense of the ripe fruit were therefore isolated from the ripe fruit after being challenged with the fungus (Oh *et al.*, 1999b, c). One of our research goals was to produce transgenic pepper plants with enhanced resistance to the anthracnose fungus by using those genes that might be involved the incompatible interaction.

We report here the isolation of the pepper TLP gene that is differentially expressed in compatible and incompatible interactions during fruit ripening. The accumulation of the *PepTLP* mRNA and *PepTLP* protein was higher in the incompatible interaction than in the compatible interaction during the early infection process. That the *PepTLP* gene transcription could be induced by wounding, but not by jasmonic acid (JA) treatment, suggested that there is a JA-independent wound signal transduction pathway in the unripe pepper fruit. The *PepTLP* mRNA level is developmentally regulated with beginning of fruit ripening, and increases upon fungal infection. Also, anthracnose development was significantly prevented from the same fruit stage. In addition, the sum total of sugar accumulation grew significantly from the early-breaker fruit. Thus, these data indicate that there is a correlation between *PepTLP* gene expression, disease resistance, and sugar accumulation with beginning of fruit ripening of the nonclimacteric pepper.

Materials and methods

Plant material, fungus, and inoculation test

Pepper (*Capsicum annuum*) cv. Nokkwang was used in this experiment. Fruits were harvested at mature-green (30 days after anthesis), early-breaker (50 days), and ripe stages (55, 58 and 65 days) at Kwangju, Korea. Samples of leaf, root, and stem were harvested from 3-week old plants. For wounding, fruits and other organs were deeply scratched with a knife and incubated under relative humidity of 100% at 27 °C for 24 h under conditions as for the inoculation test. For chemical inducer applications, 10 μ l of ethephone (1 and 10 mM), JA (4 and 40 μ M), or SA (0.5 and 5 mM) were applied to the fruit for 24 h under the above-mentioned conditions. After incubation, samples of 1 cm² were excised from the application site of the chemicals, wounding, or fungus inoculation. The samples were then frozen in liquid nitrogen.

C. gloeosporioides was cultured and harvested as described previously (Oh *et al.*, 1998). Of a spore suspension (5×10^5 spores/ml) 10 μ l was used for the inoculation of pepper fruits as described previously (Kim *et al.*, 1999).

Lesion diameter and numbers of spores on inoculated fruits were evaluated to serve as parameters of anthracnose development as described previously (Kim *et al.*, 1999). The disease parameters were recorded 7 days after inoculation.

Gene cloning, sequencing, and homology search

Modified mRNA differential display (Oh *et al.*, 1995) was performed with total RNA extracted from uninfected or infected unripe and ripe fruits 24 and 48 h after fungal inoculation. Total RNA was extracted using the RNeasy Plant kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. A partial cDNA, pddICC4, isolated by differential display screening, was used as a probe to screen a *C. gloeosporioides*-induced pepper cDNA library (Oh *et al.*, 1999c). Preliminary northern blot analysis was done to confirm the differential expression of the partial cDNA. After three rounds of plaque hybridization, a full-length cDNA was isolated and sequenced as described (Oh *et al.*, 1999b). cDNA sequencing was performed with an ALFexpress automated DNA sequencer (Amersham Pharmacia Biotech, Buckinghamshire, UK). Analysis of nucleotide and amino acid sequences was performed with the DNASIS sequence analysis software for Windows, version 2.1 (Hitachi,

San Bruno, CA). For a homology search, the cDNA sequence was compared to the sequences in the NCBI nonredundant database using the BLAST electronic mail server (Altschul *et al.*, 1997). The multiple sequence alignment was produced using the Clustal W program.

Northern, Southern, and western blots analysis

Total RNA (10 μ g/lane) from each plant tissue used in this study was separated in 1.2% denaturing agarose gels in the presence of formaldehyde. RNA gel blotting, hybridization, and washing was conducted as described by the manufacturer of the positively charged nylon membrane employed (Hybond N⁺; Amersham Pharmacia Biotech).

Genomic DNA from 4-week old plants was isolated using the DNeasy Plant Mini kit (Quiagen). Aliquots of 5 μ g DNA were digested with *Eco*RI, *Eco*RV, or *Hind*III and separated in a 0.8% agarose gel. Afterwards, DNA gel blotting and hybridization were conducted according to the manufacturer's instructions (Amersham Pharmacia Biotech). Filters were washed twice with 2 \times SSC, 0.1% SDS at 45 °C and once with 0.1 \times SSC, 0.1% SDS at 65 °C.

Radiolabeled probes were prepared with [α ³²P] dCTP (Amersham Pharmacia Biotech), using a random primer labeling kit (Boehringer, Mannheim, Germany).

SDS-PAGE was performed with total proteins separated on 15% polyacrylamide gels and electrotransferred onto PVDF membranes. The anti-TLP antibody was used at a 1:2000 dilution. A goat anti-rabbit antibody coupled to alkaline phosphatase was used as secondary antibody at a 1:5,000 dilution. The secondary antibody was visualized with luminol (ECL, USA).

Tissue preparation and immunohistochemistry

Pepper fruits were fixed in 1% glutaraldehyde/2% paraformaldehyde in 100 mM sodium phosphate buffer pH 7.0, dehydrated in ethanol, and embedded in paraffin. Tissues were transverse-sectioned into slices 10 μ m in thickness. For immunolabeling, deparaffinized sections were incubated with primary antibody for 12 h at 12 °C. Rabbit antiserum against a Chinese cabbage TLP was used at a dilution of 1:1,500 in 0.05 M Tris containing 1% BSA. Control tissues were incubated with pre-immune serum. Then the sections were washed three times with 0.05 M Tris containing 0.3 M NaCl and 0.1% Tween 20 prior

to incubation with biotinylated secondary antibody of goat anti-rabbit (DAKO, Carpinteria, CA). For detection, the secondary antibody complexed with peroxidase-conjugated streptavidin was colorized with 3-amino-9-ethylcarbazole as the substrate according to the manufacturer's instructions (DAKO).

Measurement of sugar

Thirty fruits of five different developing stages of pepper fruits were harvested from the greenhouse. The frozen samples were then ground in liquid nitrogen. Frozen powder (500 mg) was suspended in 500 μ l of ice-cold 0.05 M sodium phosphate buffer pH 7.0. After centrifugation, the supernatant was used to measure sugar contents. Fru, Glu, and Suc were assayed using a Sucrose/D-Glucose/D-Fructose Enzymatic BioAnalysis kit (Boehringer) as recommended by the manufacturer.

Results

Cloning of a fungus-inducible PepTLP gene of pepper

In previous studies, *C. gloeosporioides* interacted incompatibly with the ripe fruit of pepper, but compatibly with the unripe fruit (Oh *et al.*, 1998; Kim *et al.*, 1999). To look for a molecular mechanism involved in the incompatible interaction (Oh *et al.*, 1999b, c), we isolated genes differentially expressed in the ripe fruit, but not in the unripe fruit upon fungal infection using mRNA differential display (Liang and Pardee, 1992). Amplified cDNAs from the ripe fruit were excised from the gel, re-amplified, and cloned. In a preliminary RNA gel blot analysis, a cDNA clone, named pddICC4 for incompatible *Capsicum annuum*/*Colletotrichum gloeosporioides* interaction, hybridized to a transcript of 0.95 kb that accumulated in the incompatible interaction (data not shown). DNA gel blot analysis under high-stringency conditions with the pddICC4 as the probe revealed one band in each enzyme digest, suggesting that pddICC4 is a single-copy gene in the pepper genome (Figure 1).

To isolate the full-length cDNA clone, the insert of pddICC4 was used as probe for plaque hybridization with a cDNA library prepared from fruits 24 and 48 h after inoculation (HAI) with the fungus. A clone containing the longest insert was designated PepTLP (pepper thaumatin-like protein), isolated, and sequenced. The 3' region of the PepTLP clone contained the nucleotide sequence of pddICC4 as expected.

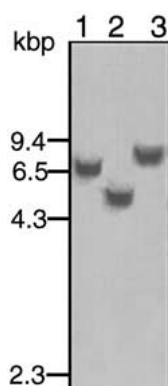


Figure 1. A pepper genomic blot probed with a *PepTLP* gene-specific probe, pddlCC4. The restriction enzymes are *EcoRI* (1), *HindIII* (2), and *EcoRV* (3).

Sequence analysis and characterization of *PepTLP* cDNA

The *PepTLP* cDNA is 937 bp in length with 8 bp of 5'-untranslated region and 191 bp of 3'-untranslated region including the poly(A) tail (GenBank AF297646). The *PepTLP* clone thus represents the full-length cDNA of the 0.95 kb transcript identified by RNA gel blot analysis before. The deduced amino acid sequence of *PepTLP* contains an N-terminal secretory signal peptide that is cleaved after alanine at position 21 (Figure 2). The cDNA except the signal peptide contains one open reading frame encoding a polypeptide of 225 amino acids with a predicted molecular mass of 24 kDa and an isoelectric point of 7.5. Also, the deduced amino acid sequence contains 16 conserved cysteine residues and the consensus pattern of thaumatin, GRGSCQTGDCGGVLQC, at amino acid positions 78–93.

Sequence alignment showed that the *PepTLP* was 91, 88, 87, 60, and 48% identical to the TLPs of potato (Zhu *et al.*, 1993), tomato (King *et al.*, 1988), tobacco (Neale *et al.*, 1990), grape berry (Tattersall *et al.*, 1997), and banana (Clendennen and May, 1997), respectively (Figure 2). These TLPs are expressed in response to various environmental and developmental stimuli. The potato TLP is induced during freezing tolerance. The tobacco TLP is induced during flowering and by abiotic (Singh *et al.*, 1989) and biotic stresses (Woloshuk *et al.*, 1991). In addition, although the tomato TLP is induced by salt stress (King *et al.*, 1988), the cherry and banana TLPs as well as the tomato TLP (Pressey, 1997) are expressed during fruit ripening.

Differential induction during compatible and incompatible interactions

In our previous studies (Oh *et al.*, 1998; Kim *et al.*, 1999), higher levels of appressorium and infection hypha formation were observed on the unripe fruit than on the ripe fruit at 12 h and 24 h after inoculation (HAI), respectively. After that, initial anthracnose symptoms were detected only on the unripe fruit after 48 HAI, and typical sunken necrosis occurred within 120 HAI. We then examined whether the time-course of the accumulation of *PepTLP* mRNA after *C. gloeosporioides* inoculation correlated with fungal morphogenesis and symptom development. RNA gel blot analysis was performed on both unripe and ripe fruits at 0, 3, 6, 12, 24, 48, and 72 HAI. In the unripe fruit, a basal level of *PepTLP* mRNA was not detected, but some accumulation of *PepTLP* mRNA was detected at 3 HAI (Figure 3). The expression of *PepTLP* mRNA was low at 3, 6, 24, and 48 HAI, but a biphasic accumulation of *PepTLP* mRNA reached a maximum at 12 and 72 HAI. In contrast, in the ripe fruit, a basal level of *PepTLP* mRNA was detected and reached a maximum already at 3 HAI, and the expression level remained elevated. Water inoculation without fungal spores as a mock test did not significantly induce the accumulation of *PepTLP* mRNA in both fruit types.

Immunolocalization and immunoblot analysis of *PepTLP* during early infection

To examine the localization and accumulation of *PepTLP* protein during early infection, we performed immunolocalization experiments using antibodies to Chinese cabbage TLP and sections. The transverse-sections were prepared from the infection sites of both ripe and unripe fruits at 72 HAI. The *PepTLP* was not detectable in the intercellular spaces of cortical cells in the uninoculated unripe fruit (Figure 4A-a). However, it was detected in those spaces in the uninoculated ripe fruit (Figure 4A-b). When the fungus had colonized the outer epidermal cells of the unripe fruit at 72 HAI (Kim *et al.*, 1998), the protein was significantly induced (Figure 4A-c). And, although fungal invasion was rarely observed in the ripe fruit at 72 HAI, the *PepTLP* was prominently present in the intercellular spaces among cortical cells (Figure 4A-d). Control fruit sections were treated with pre-immune serum (Figure 4A-a' to 4A-d').

In addition, immunoblot analysis showed that the *PepTLP* (23 kDa) accumulated in the ripe fruit, but not in the unripe fruit (Figure 4B). The accumulation

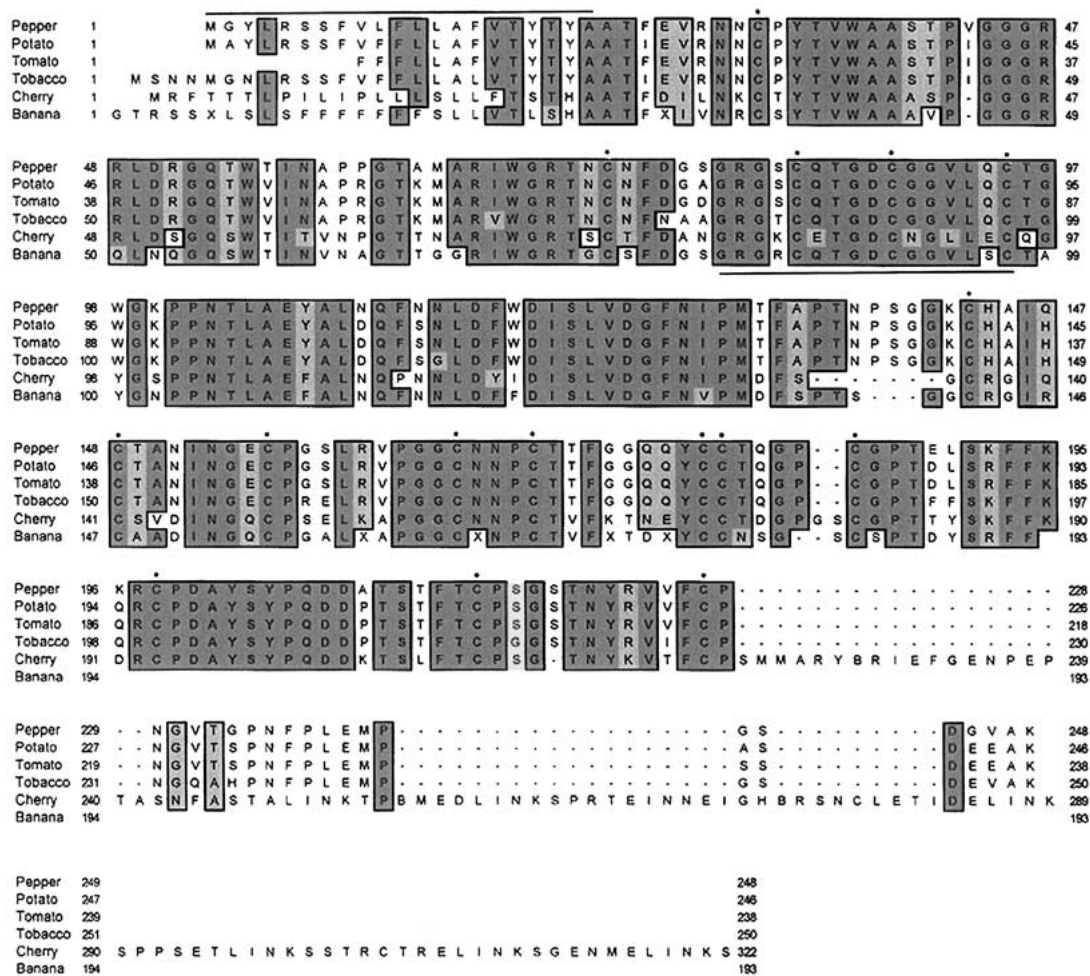


Figure 2. Alignment of the deduced amino acid sequence from *PepTLP* cDNA (pepper) (GenBank accession number AF297646) with other TLPs from potato (P50701), tomato (PI2670), tobacco (S30157), grape berry (grape) (AAB61590), and banana (AAB82777). The upper line demarks the hydrophobic N-terminal membrane anchor region of *PepTLP* (amino acid residues 1–23). The deduced amino acid sequence contains 16 conserved cysteine residues indicated by the dots. The consensus pattern of thaumatin, GRGSCQTGDCGGVLQC, between amino acids 78 and 93 is indicated by the underline.

of *PepTLP* protein after fungal infection was higher in the ripe fruit than in the unripe fruit.

Expression patterns upon fungal infection, wounding, and by chemical inducers

To examine *PepTLP* gene expression in various organs after fungal inoculation or wounding, RNA gel blot analyses were performed on total RNA prepared from fruits, leaves, stems, and roots of pepper plants 24 h after treatment. In the fruit, a basal expression of the *PepTLP* gene was observed only in the ripe fruit. But *PepTLP* mRNA accumulated to high levels in both fruits upon fungal infection or wounding (Figure 5A). The accumulation of the *PepTLP* mRNA increased

also in the stem after fungal infection or wounding, but in the leaf only upon wounding. In the root, a basal level of *PepTLP* mRNA was detected, but this level of *PepTLP* mRNA did not significantly change after either treatment.

To examine chemical inducers of *PepTLP* gene expression in fruit, RNA gel blot analysis was performed on total RNAs prepared from the application sites of both ripe and unripe fruits of drop-applied ethephone, jasmonic acid (JA), or salicylic acid (SA) after 24 h. The *PepTLP* mRNA accumulated highly only in the ripe fruit treated with JA at 40 M (Figure 5B), while SA and ethephone did not affect the *PepTLP* gene expression in either the ripe or unripe fruit. To test

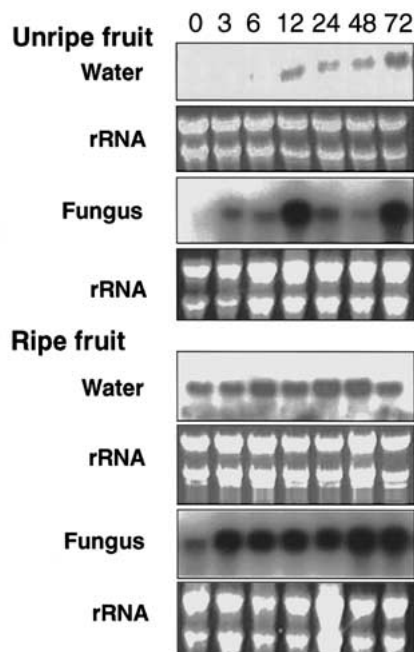


Figure 3. Differential induction of the *PepTLP* gene in pepper fruits upon *Colletotrichum gloeosporioides* inoculation. Total RNAs were isolated from both unripe and ripe fruits after fungal inoculation (Fungus) on a time-course. A water mock inoculation (Water) without fungal spores of both unripe and ripe fruits was used as a control. The RNAs were electrophoresed and allowed to hybridize with pddICC4 (*PepTLP*). Numbers indicate hours after inoculation.

whether a high concentration of JA would be able to induce the expression of *PepTLP* in the unripe fruit, JA was applied to the unripe fruit at up to 1 mM. No induction of *PepTLP* expression was observed in the unripe fruit treated with JA (data not shown).

Expression during ripening stages

To examine the expression of the *PepTLP* gene during the ripening process of the pepper fruit, RNA gel blot analysis was performed using total RNAs prepared at five stages of fruit development: unripe-mature green, early-breaker, turning, purple, and ripe-red. *PepTLP* mRNA was detected in the early-breaker fruit, and the accumulation of *PepTLP* mRNA slightly increased during ripening (Figure 6). We further examined *PepTLP* gene expression after fungal infection, JA, or wounding during fruit ripening. The *PepTLP* mRNA accumulated to low levels in the unripe fruit at 3 HAI. However, the accumulation after fungal infection was much elevated over the early-breaker stage of the fruit and continued to increase during ripening. Wounding also led to the accumulation of *PepTLP* mRNA in the

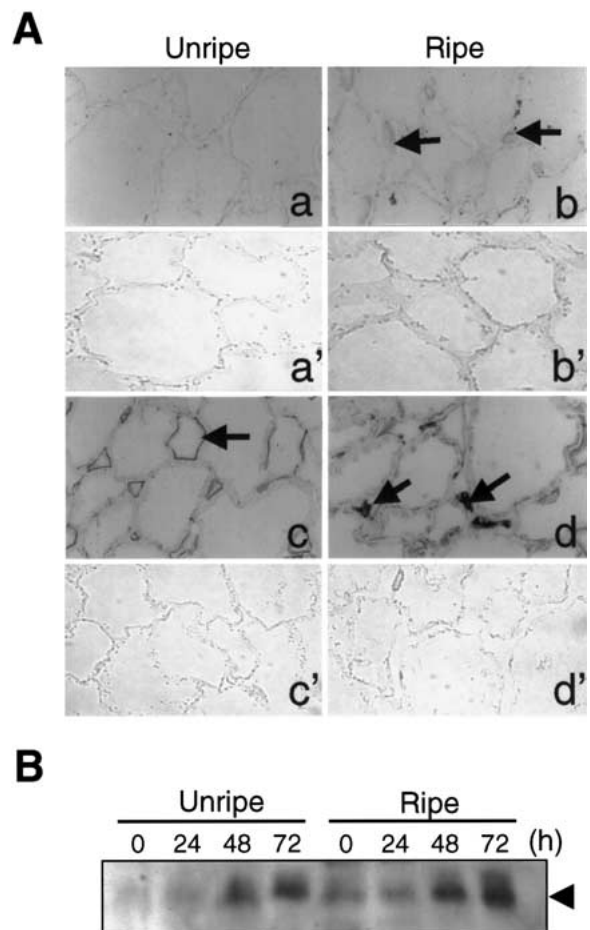


Figure 4. A. Immunolocalization of *PepTLP* protein in pepper fruits using an antibody to Chinese cabbage TLP. The *PepTLP* was not detectable in the intercellular spaces of cortical cells of uninoculated unripe fruits (a), but they were detected in those spaces of uninoculated ripe fruits (b). The *PepTLP* was significantly induced in both the unripe (c) and ripe fruit (d) at 72 h after inoculation with *Colletotrichum gloeosporioides*. The arrow indicates accumulated *PepTLP*. Control fruit sections treated with pre-immune serum are a' to d'. B. Immunoblot analysis of *PepTLP* in the unripe and ripe pepper fruits inoculated with *C. gloeosporioides*. The blot was incubated with antiserum against Chinese cabbage TLP. The accumulation of *PepTLP* protein (23 kDa) after fungal infection was higher in the ripe fruit than in the unripe fruit.

unripe fruit up to the ripe fruit stage, and the accumulation was highly accelerated between the turning stage and the ripe fruit stage. Upon JA application, the *PepTLP* mRNA highly accumulated from the early-breaker fruit up to the ripe fruit stage, but not in the unripe fruit. The accumulation was not significantly different during fruit ripening stages.

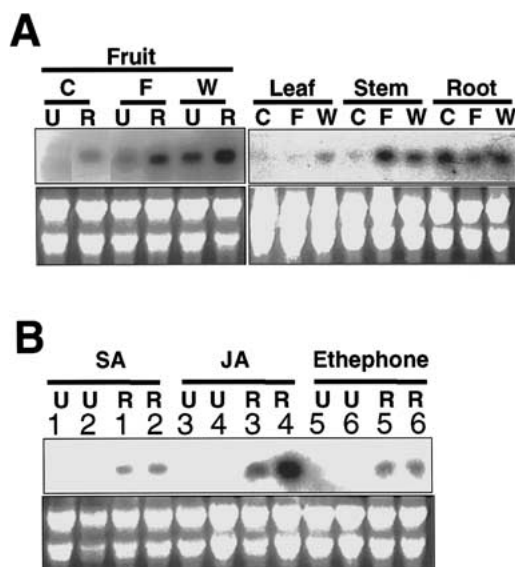


Figure 5. A. Expression and induction of the *PepTLP* gene from various organs of pepper by *Colletotrichum gloeosporioides* inoculation or wounding. Total RNAs were isolated from the application sites of unripe fruit (U), ripe fruit (R), leaf, stem, and root 24 h after treatments consisting of fungal inoculation (F) or wounding (W). Aliquots of 10 μ l (5×10^5 spores per ml) were used for drop inoculation of various pepper organs. Organs treated with 10 μ l of sterile water containing no fungal spores were used as controls (C). The RNAs were electrophoresed and probed with pddICC4 for *PepTLP*. B. Induction of the *PepTLP* gene in unripe and ripe fruits of pepper after exogenous application of salicylic acid (SA), jasmonic acid (JA), and ethephone treatment. Total RNAs were isolated from the application sites of both unripe (U) and ripe fruit (R). The drop inoculations consisted of 10 μ l of SA (1 = 0.5 mM, 2 = 5 mM), JA (3 = 4 μ M, 4 = 40 μ M), and ethephone (5 = 1 mM, 6 = 10 mM). The RNAs were electrophoresed and probed with pddICC4 for *PepTLP*.

Anthracnose development on pepper fruits during ripening stages

To examine the relationship between ripening stages and anthracnose development, five different developmental stages of fruit were drop-inoculated with a spore suspension of *C. gloeosporioides*. Typical anthracnose symptoms with necrotic and sunken lesions were observed on the unripe fruit within 5 days after inoculation, just as we had previously observed (Oh *et al.*, 1998) (Figure 7). No anthracnose symptoms were observed on the ripe fruit inoculated with the fungus. Anthracnose disease development was measured in terms of lesion diameter and number of fungal spores produced from the lesions. Anthracnose symptoms were significantly prevented with beginning of ripening. The lesion in the early-breaker fruit was similar to that of very beginning of typical anthrac-

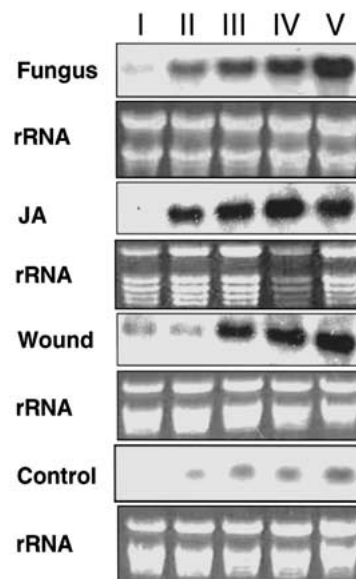


Figure 6. Expression and induction of the *PepTLP* gene at different developmental stages of the pepper fruit upon fungal inoculation (Fungus), JA application, or wounding. Total RNAs were isolated from the application sites 24 hrs after treatment with JA or wounding, and only 3 h after inoculation with the fungus. A 10 μ l portion of a 5×10^5 spores/ml suspension was used for drop inoculation. Fruits treated with 10 μ l of sterile water without fungal spores were used as controls (Control). The RNAs were electrophoresed and probed with pddICC4 for *PepTLP*. I, mature-unripe green fruit; II, early breaker fruit; III, turning fruit; IV, purple fruit; V, ripe red fruit.

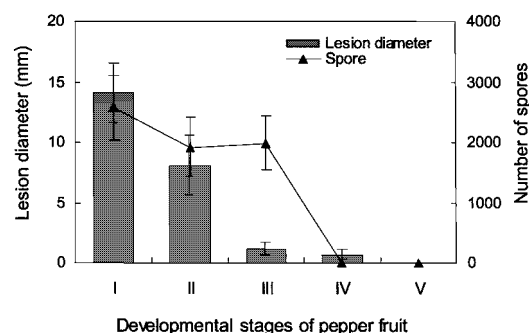


Figure 7. Diameter and spore production of anthracnose lesions caused by *Colletotrichum gloeosporioides* on pepper fruits at different developmental stages. The unripe fruit (I) exhibited typical sunken necrotic symptoms, but the ripe fruit (V) showed no such symptoms. Anthracnose disease development was significantly lower in the turning fruit (III). Each value for lesion diameter and number of spores represents a mean of 30 and 15 observations, respectively (\pm SE). I, mature-unripe green fruit; II, early breaker fruit; III, turning fruit; IV, purple fruit; V, ripe red fruit.

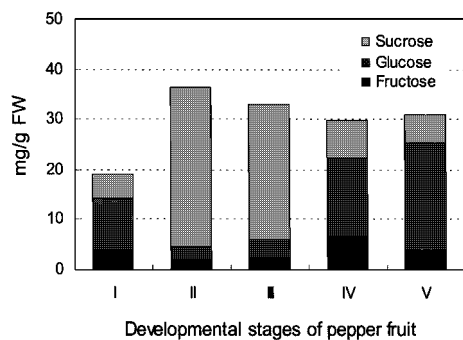


Figure 8. Each amount and the sum total of fructose, glucose, and sucrose at different developmental stages of pepper fruits. I, mature-unripe green fruit; II, early breaker fruit; III, turning fruit; IV, purple fruit; V, ripe red fruit. Data are the means of three independently performed experiments.

nose symptoms (data not shown). However, the lesion was arrested and not developed further. Lesion development was greatly arrested in the turning fruit, although spores were still produced. In the purple fruit, both lesion development and spore production were prevented.

Sugar accumulation of pepper fruits during different ripening stages

Accumulation of Fru, Glu, and Suc were measured at different developmental stages of pepper fruits. Suc accumulation significantly increased between the unripe and the early breaker stage. Then it slightly decreased in the turning fruit (Figure 8). However, in the purple and ripe fruits, Suc accumulation was much lower than that in the early breaker and turning fruits. In contrast to the Suc accumulation, the amounts of Fru and Glu at the early breaker and turning fruit stages were lower than in the unripe fruit. However, Fru and Glu were increased in the purple fruit. Finally, Glu accumulation was elevated in the ripe fruit, but Fru accumulation was decreased in the ripe fruit. This result indicated that the sum total of Fru, Glu, and Suc was highly accumulated with beginning of fruit ripening.

Discussion

Fruit ripening has been mainly studied in climacteric fruit, and relatively little is known about molecular mechanisms of the ripening of nonclimacteric fruit, such as cherry (Fils-Lycaon *et al.*, 1996) and grape berry (Tattersall *et al.*, 1997; Salzman *et al.*, 1998).

Especially, there have been few studies of disease susceptibility/resistance in relation to the ripening of nonclimacteric fruits. Recently, we reported anthracnose development on the unripe fruit of pepper, but not on the ripe fruit (Oh *et al.*, 1998; Kim *et al.*, 1999). This complex pathosystem may generate significant information towards the understanding of the interaction between fruit development and disease resistance in plants. To investigate the effect of fruit maturity on the ability of *C. gloeosporioides* to develop an infection, we examined in this study *PepTLP* gene expression, disease resistance by inoculation test, and sugar content at different developmental stages of the pepper fruit. With beginning of fruit ripening, a rise in the expression of the *PepTLP* gene upon fungal infection, a drop in the incidence of anthracnose disease, and an accumulation of sugar were observed. This study shows a correlation between *PepTLP* gene expression, disease resistance, ripening, and sugar accumulation in fruits. Thus, the *PepTLP* gene may be used as a molecular marker to probe disease resistance, ripening, and sugar accumulation in nonclimacteric pepper fruits.

In a pepper fruit/anthracnose fungus interaction, the expression of the *PepTLP* gene in response to fungal infection was temporally and spatially regulated during incompatible and compatible interactions. Transcript levels in the two kinds of interactions were very different, with a weak biphasic accumulation in the compatible interaction and maintenance of elevated levels in the incompatible interaction. During a previous study, a pepper thionin-like protein gene, *PepThi* (Oh *et al.*, 1999c), and a pepper cytochrome P450 gene, *PepCYP* (Oh *et al.*, 1999b), genes that were highly expressed only during an incompatible interaction, had been cloned. This differential expression has also been reported for other plant-pathogen interactions (Ebrahim-Nesbat *et al.*, 1993; Lin *et al.*, 1996; Gregersen *et al.*, 1997).

In an observation of fungal morphogenesis, *C. gloeosporioides* started to germinate at 3 HAI, and appressorium formation was observed at 12 HAI (Oh *et al.*, 1998; Kim *et al.*, 1999). In this study, expression of the *PepTLP* gene was detected on both unripe and ripe fruits at 3 HAI at the time when the fungus germinates. This result suggests that the *PepTLP* gene is induced in fruits by fungal elicitors. On the other hand, the *PepThi* and *PepCYP* genes were expressed in the fruits at 12 and 24 HAI, respectively, indicating that these two genes are expressed when the fungus starts to penetrate the plants. Taken together, these observa-

tions suggest that the plant deploys different defense mechanisms in response to different stages of fungal morphogenesis during the early infection process.

In an immunolocalization study, we found the PepTLP protein localized in the intercellular spaces of cortical cells. This is consistent with the nature of PepTLP, since it has an N-terminal secretory signal peptide. Once *C. gloeosporioides* successfully penetrated into the unripe fruit, the fungus invaded and colonizes other cells via the intercellular spaces and vascular bundles (unpublished data). Since no colonization of the fungus was observed in the ripe fruit, these data suggest that the fruit is protected against fungal colonization because of the presence of PepTLP in these intercellular spaces in the ripe fruit.

Evidence of developmental regulation of TLPs during fruit ripening has been found in banana (Clenndennen and May, 1997), cherry (Fils-Lycaon *et al.*, 1996), grape berry (Salzman *et al.*, 1998), and tomato (Pressey, 1997). In addition, TLPs were found to accumulate in tobacco flowers (Richard *et al.*, 1992) and in maize seeds (Roberts and Selitrennikoff, 1990). Antifungal activity has been suggested as a common function of TLPs (Roberts and Selitrennikoff, 1990; Vigers *et al.* 1992). In this study, the expression of the *PepTLP* gene was developmentally regulated during ripening and, in particular, elevated in the ripe fruit in response to fungal infection. Thus, the data strongly suggest that TLPs play a significant role in the protection of reproductive organs. In a previous study, *PepThi* and pepper defensin (*j1-1*; Meyer *et al.*, 1996) were expressed during pepper fruit ripening (Oh *et al.*, 1999c). Thus, the concerted expression of several defense genes, including *PepTLP*, *PepThi*, *j1-1*, and *PepCYP*, may confer disease resistance of the ripe fruit against *C. gloeosporioides* infection.

The TLP gene is not only developmentally regulated but also controlled by biotic or abiotic stress, such as cold (Zhu *et al.*, 1993) and salt (Kononowicz *et al.*, 1992). In addition, a TLP gene is induced in *Solanum commersonii* by abscisic acid (ABA) and wounding (Zhu *et al.*, 1993), and in tobacco seedlings by ethylene, methyl JA, and SA (Xu *et al.*, 1994). However, the *PepTLP* gene was induced only in the ripe pepper fruit by JA, while it could be induced in both the unripe and the ripe fruit by wounding (Figure 5). It is likely that the *PepTLP* gene expression is regulated in the unripe fruit via a JA-independent wound signal transduction pathway. In *Arabidopsis*, a JA-independent wound signaling pathway that was oppositely regulated to a JA-dependent pathway, has

been identified (Rojo *et al.*, 1998). These data indicate that there could be two different signaling pathways stimulated by wounding during the pre- and post-ripening stages of the pepper fruit. This is consistent with the response of fruits during ripening processes against wounding or JA treatment (Figure 6).

Both ethylene and ABA are known to have significant roles in the ripening process of climacteric fruits in which they induce PR proteins including TLP (Xu *et al.*, 1994). In contrast, it is uncertain what plant chemical signals are involved in the ripening of nonclimacteric fruit. It has been suggested that the TLP induction in nonclimacteric berry fruits occurs by another SA-independent mechanism (Tattersall *et al.*, 1997). The *PepTLP* gene was highly induced from the early breaker stage by JA. Still, there is little known about the role of JA in nonclimacteric fruit. Only a few cases in which methyl JA triggered the ripening process in tomato and apple with ethylene production have been described (Saniewski *et al.*, 1987a, b; Czapski and Saniewski, 1992).

The sum total of Suc, Fru, and Glu significantly accumulated with beginning of ripening. This is consistent with disease resistance enhanced from the early-breaker fruit. Vanderplank (1984) suggested that higher levels of sugar exert an osmotic stress on fungi, thus protecting the plant from fungal colonization. In particular, the efficacy of the antifungal protein activity against phytopathogens of the nonclimacteric grape berry fruit was enhanced by higher concentrations of sugar (Salzman *et al.*, 1998). In addition, although there are many examples of sugar repressed genes, sugars also induce a number of defense-related genes, such as chalcone synthase (Tsukaya *et al.*, 1991), PR genes (Herbers *et al.*, 1995), and proteinase inhibitor II (Johnson and Ryan, 1990). Taken together, these data suggest that a concerted mechanism of sugar accumulation, enhancement of antifungal protein activity, and defense-related gene expression induced by sugar enhances disease resistance of the fruit against phytopathogens with beginning of ripening.

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